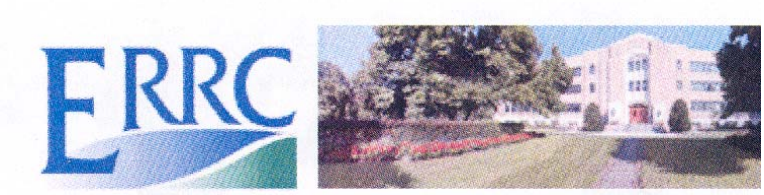

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Title: Applications of Immunomagnetic Capture and Time-Resolved Fluorescence to Detect Outbreak *Escherichia coli* O157 and *Salmonella* in Alfalfa Sprouts

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Citation: In "Monitoring Food Safety, Agriculture, and Plant Health, ed by B.S. Bennedsen, Y.R. Chen, G.E. Meyer, A.G. Senecal, and S.I. Tu. Proceeding of Spie Volume 5271 (Spie, Bellingham, WA 2004) (2004) Page 7-12

Number: 7356

Applications of Immunomagnetic Capture and Time-Resolved Fluorescence to Detect Outbreak *Escherichia coli* O157 and *Salmonella* in Alfalfa Sprouts

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ABSTRACT

Commercially available alfalfa seeds were inoculated with low levels (~ 4 CFU/g) of pathogenic bacteria. The inoculated seeds were then allowed to sprout in sterile tap water at 22 °C. After 48 hours, the irrigation water and sprouts were separately transferred to bovine heart infusion (BHI) media. The microbes in the BHI samples were allowed to grow for 4 hours at 37 °C and 160 rpm. Specific immunomagnetic beads (IMB) were then applied to capture the *E.coli* O157 and/or *Salmonella* in the growth media. Separation and concentration of IMB-captured pathogens were achieved using magnetic separators. The captured *E. coli* O157:H7 and *Salmonella spp* were further tagged with europium (Eu) labeled anti-*E. coli* O157 antibodies and samarium (Sm) labeled anti-*Salmonella* antibodies, respectively. After washing, the lanthanide labels were extracted out from the complexes by specific chelators to form strongly fluorescent chelates. The specific time-resolved fluorescence (TRF) associated with Eu or Sm was measured to estimate the extent of capture of the *E. coli* O157 and *Salmonella*, respectively. The results indicated that the approach could detect *E. coli* O157 and *Salmonella* enterica from the seeds inoculated with ~ 4 CFU/g of the pathogens. Non-targeted bacteria, e.g., *Aeromonas* and *Citrobacter* exhibited essentially no cross reactivity. Since the pathogen detection from the sprouts was achieved within 6 hours, the developed methodology could be use as a rapid, sensitive and specific screening process for *E. coli* O157 and *Salmonella* enterica in this popular salad food.

Keywords: Bacterial detection, *E. coli* O157:H7, *Salmonella spp.*, Time Resolved Fluorescence, Immunomagnetic beads, Alfalfa sprouts, Lanthanide Cations.

1. INTRODUCTION

The use of plant sprouts as a salad ingredient has gained considerable acceptance by American consumers. However, outbreaks of *E. coli* O157:H7 and *Salmonella* spp. associated with the consumption of raw sprouts have become a concern [1, 2, 3]. Sprouting of pathogen contaminated seeds may lead to rapid growth of the bacteria to levels as high as $10^6 - 10^8$ CFU/g of product [4, 5] and the internalization of bacteria into the edible parts of the spouts, makes them difficult to disinfect after sprouting [6, 7].

Direct testing of contaminated seeds is difficult because of the unevenness distribution of pathogens [8]. Thus, the Food and Drug Administration has recommended testing of spent irrigation water from sprout production instead [9]. Currently, FDA [9] recommends the use of VIP EHEC (Biocontrol Systems, Inc., Bellview, WA) or Reveal *E. coli* O157:H7 tests (Neogen Corp., Lansing, MI) for the detection of *E. coli* O157:H7 and Assurance Gold *Salmonella* EIA or Visual Immuno-precipitate (VIP) assay for *Salmonella* (both from Biocontrol Systems, Inc., Bellview, WA) for the detection of *Salmonella* spp in the spent irrigation water. However, the *E. coli* tests require an overnight incubation in modified buffered peptone water with three added antibiotics and the *Salmonella* methodology requires pre-enrichment and enrichment for approximately 48-50 hours before testing. Thus, there is a need to develop sensitive and specific alternatives that can be completed in shorter time periods.

Recently, we have combined the immunomagnetic beads (IMB) technique with time-resolved fluorescence (TRF) of lanthanide cations, to detect *E. coli* O157:H7 and *Salmonella* in foods [10, 11, 12]. The long fluorescence half-lives

and considerable Stoke's shifts between the absorption and emission maxima of La cations minimize both the background fluorescence interference and random scattering of excitation light. Consequently, utilizing pulsed excitation, the fluorescence of La may be easily filtered out from the interference fluorescence and scattered excitation light by delaying the emission measurement. The developed process is capable of detecting very low levels of *E. coli* O157:H7 and *Salmonella* spiked in ground meats and in apple cider and has been found to show a minimal cross-reactivity with non-target bacteria. We have modified and extended the developed procedure to detect *E. coli* O157 and *Salmonella* in both 48 h spent alfalfa sprout irrigation water and germinated alfalfa sprouts. The results demonstrated that selected outbreak strains of *E. coli* O157:H7, *E. coli* O157:NM and *Salmonella* found in sprouts, were detectable in both the spent irrigation water and sprouts germinated from contaminated seeds (~ 4 CFU/g) after a brief enrichment for 4 h at 37 C.

2. MATERIALS AND METHODS

2-1. Collection of Pathogens from Alfalfas Germinated from Pathogen Inoculated Seeds.

Fresh *E. coli* and *Salmonella* cultures were used to inoculate alfalfa seeds in growth-jars with indicated dosages. Twenty-four hours after the seeds were inoculated, sterile tap water was added to each jar, the jars were swirled for 15 seconds, the spent irrigation water was decanted and the jars were returned to storage in the closed plastic container. At 48 h, sterile tap water was added to each jar, swirled as above to mix and 9.0 mL of the water transferred to a 15 mL sterile conical tube containing 1.0 mL of 10X BHI. The tubes were then incubated for 4 h at 37 C and 160 rpm. To test the sprouts for pathogens, at 48 hours of germination, normal strength BHI in a volume of 250 mL was added to duplicate jars with growing sprouts. The whole sprouts plus the added media were then incubated for 4 h at 37 C and 160 rpm for bacterial enrichment.

2-2. Capture of *E. coli* and *Salmonella*

At the end of the enrichment, broth was removed from each tube (for irrigation water) or jar (for whole sprouts) to micro centrifuge tubes containing either anti-*E. coli* O157 antibody-coated IMB (Dynal Biotech, Oslo, Norway), for *E. coli* controls and *E. coli* inoculated sprouts or anti-*Salmonella* antibody-coated IMB (Dynal Biotech, Oslo, Norway), for *Salmonella* controls and *Salmonella* inoculated sprouts. The tubes were then incubated at room temperature for 20 min on a Speci-Mix rocker (Barnstead/Thermolyne, Dubuque, IA) to allow formation of bacterial-bead complexes. After the incubation, the beads were concentrated utilizing a magnetic particle concentrator (MPC, Dynal, Biotech, Oslo, Norway) and washed twice with washing concentrate (PerkinElmer Wallac, Turku, Finland) containing a Tris-HCl salt solution buffered to pH 7.8, Tween 20 and a preservative, diluted according to manufacturer's directions and supplemented with an additional 0.5% (v/v) Tween 20 (SIGMA, St. Louis, MO).

2-3. Labeling of *E. coli* and *Salmonella*

After the bacteria were captured by the beads, either europium-labeled anti-*E. coli* O157 antibody or samarium-labeled anti-*Salmonella* antibody or both (Kirkegaard & Perry Laboratories, Gaithersburg, MD; labeled at PerkinElmer Life Sciences, Norton, OH) diluted 1:1000 in TRF assay buffer (PerkinElmer Wallac, Turku Finland), supplemented with 0.1% (v/v) Tween 20 to minimize non-specific binding, was added to each tube. The tubes were then vortexed to mix and incubated at room temperature for 40 min on a Speci-Mix rocker to allow formation of bead, bacteria and lanthanide-labeled antibody sandwiches. The beads were concentrated again and washed twice as previously described.

2-4. Detection of *E. coli* and *Salmonella*

To generate highly fluorescent Eu or Sa complexes, the bead, bacteria and lanthanide-labeled antibody sandwiches were re-suspended in enhancement solution (PerkinElmer Wallac, Turku, Finland) containing Triton X-100, acetic acid and chelates, added to separate wells of a 96-well black micro plate and incubated for 5 min at room temperature on a plate shaker. Dissociation enhanced fluorescence was measured with a VICTOR^2 1420 Multilabel Counter (PerkinElmer Wallac, Turku, Finland).

3. RESULTS AND DISCUSSION

3.1. IMB-TRF for the Detection of Outbreak Strains of *E. coli* O157 and *Salmonella*

Previously, we have demonstrated that the combination of IMB and TRF will detect both *E. coli* O157:H7 and *Salmonella* spiked in ground meats with inoculum levels as low as 10^0 CFU/g [11]. This approach, as summarized in Figure 1, has yet to be tested for its applicability in detecting similar pathogens in foods of plant origins.

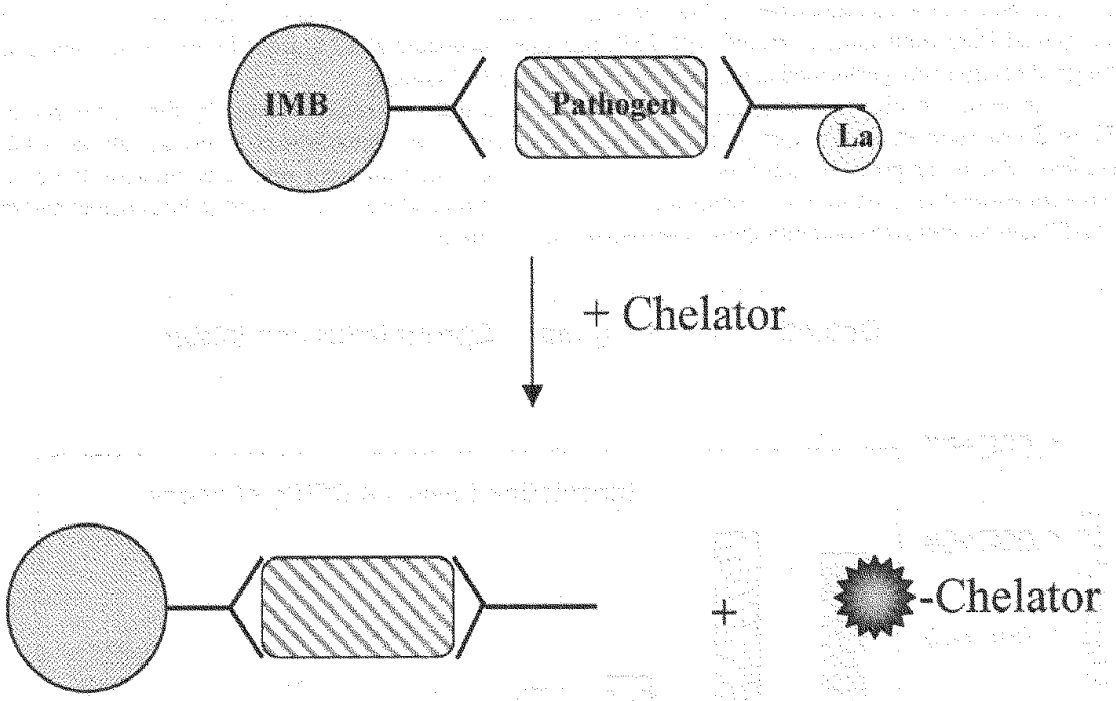

Figure 1. General Approach of IMB Capture and TRF Measurement for Pathogen Detection. Pathogens (*E. coli* O157:H7 and *Salmonella*) were captured by specific IMBs. The capture bacteria then formed sandwiched complexes with La-labeled antibodies (Eu for anti-*E. coli* O157 and Sm for anti-*Salmonella*, respectively). The sandwiches were then treated with strong chelators to extract La to form highly fluorescent products.

Outbreak strains of *E. coli* O157 and *Salmonella* cultured in our lab, were used to inoculate alfalfa seeds, allowed to multiply on the sprouting plants, captured from 48 h irrigation water or sprouts by IMB and detected by TRF (Figure 1). For pure cultures, the detection sensitivity for the *E. coli* O157 is ~ 10^3 CFU/mL. For *Salmonella*, the process can detect ~ 10^3 CFU/mL Bredeney 3VIPHE but requires 10^4 CFU/mL of other strains of *Salmonella* (Table 1).

Table 1. Detection Sensitivity toward Outbreak Stains of Pathogens*

	E. coli O157		*Salmonella*					
CFU/ML	H7 F4546	NM 98A06026	Bredeney 3VIPHE	Muenchen HERV2C	Stanley H0558	Anatum 4317	Infantis F4319	Newport H1275
1.00E+05	2691353	2803650	27044	10107	12052	13807	19479	7490
1.00E+04	868448	1203311	18631	4672	7759	3699	6023	4992
1.00E+03	63280	93134	5320	1281	3429	1301	1509	1781
0	~4500		~1500					

* The background TRF signals for the IMB and Eu-labeled and Sm-labeled antibodies used for *E. coli* O157 and *Salmonella* detection were ~4500 and 1500 CPS, respectively.

The exact reason for this observation is unknown. One possible cause may be better binding of the anti-*Salmonella* antibody to the Bredeney stain. Overall, the detection sensitivity for *E. coli* O157 is about one order of magnitude better than that for *Salmonella.*

3-2. Detection of Pathogens in Laboratory Cultivated Sprouts Grown From Inoculated Seeds

An analysis of spent irrigation water should indicate whether the sprouts, and therefore the seeds, are contaminated by pathogens. Experimentally, alfalfa seeds artificially contaminated with *E. coli* O157 or *Salmonella* were used to produce sprouts as described in Materials and Methods. Forty-eight hours after seedling, spent irrigation water was removed from each sample, mixed with BHI, and then incubated at 37 C for 4 hours. After this brief enrichment, pathogen detection was performed using described IMB-TRF technology.

As shown in Figure 2, utilizing 48 h spent irrigation water as recommended by the [9], the presence of *E. coli* O157 or *Salmonella* in alfalfa sprouts germinated from seeds with contaminated levels as low as 4 CFU/g is easily detectable. Since our previous study on ground meats demonstrated no cross reactivity between the *E. coli* O157 and *Salmonella* antibodies used in our developed procedure [11] the procedure may be used to both detect and differentiate *E. coli* O157 and *Salmonella* contamination of sprouted food products.

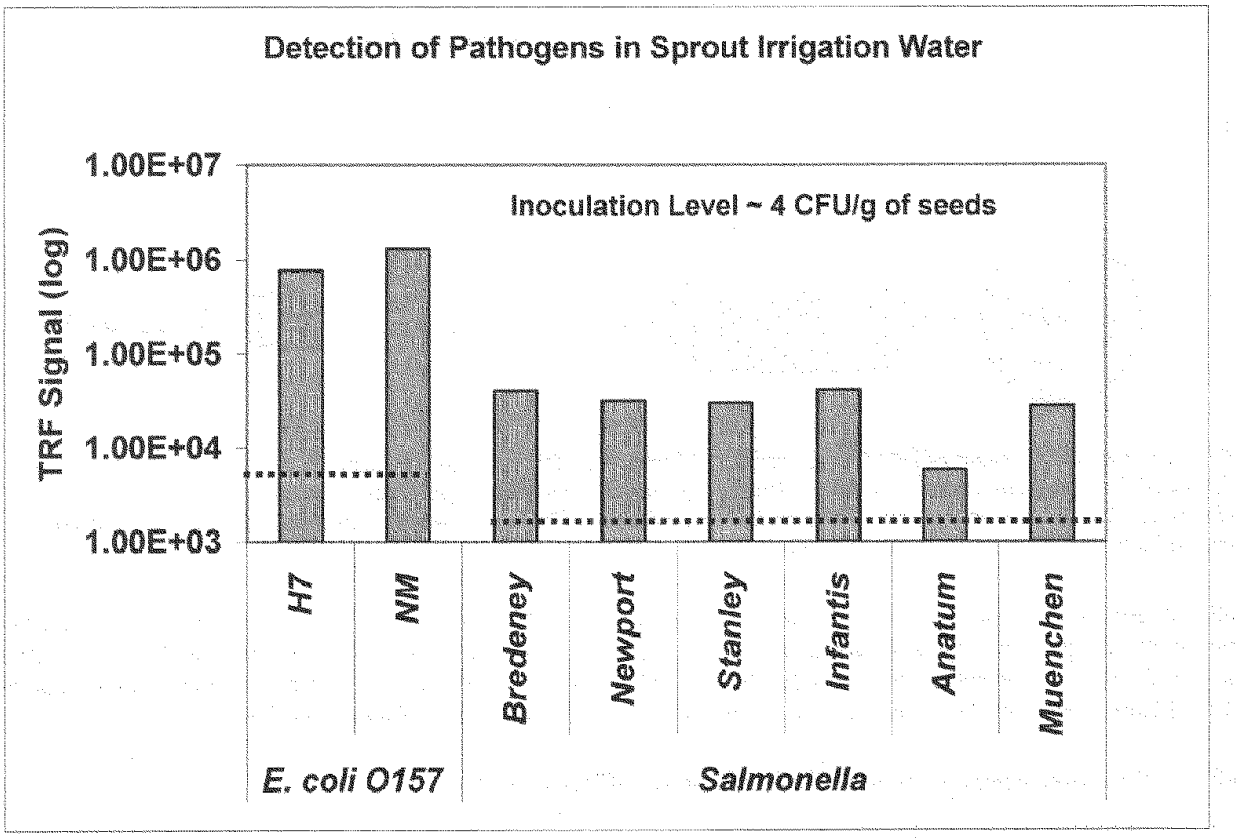

Figure 2. Detection of Pathogens in Irrigation Water. Commercially available alfalfa seeds were spiked with known level of pathogens (4 CFU/g). Contaminated seeds were allowed to germinate by irrigating with sterilized tap water. The water was collected and tested as described in Material and Methods. The data shown represent average of two experiments with probable errors as ± 10%. The dashed lines indicate the averages of background readings for *E. coli* O157 bacteria (Eu-label) and *Salmonella* bacteria (Sm-label).

The developed detection method was also applied sprouts directly. Portions of 25 g of whole sprouts were aseptically transferred to the BHI culture medium for the enrichment. Both the sprouts and the spent irrigation water equally showed the presence of the pathogens as depicted in Figure 3. This experiment demonstrated that the spent irrigation water test is indicative of the presence of *E. coli* O157 or *Salmonella* on alfalfa sprouts.

3-3. Cross reactivity and Specificity of the IMB-TRF process

In ground meat systems, the developed detection method has exhibited no cross-reactivity between *E. coli* O157 and *Salmonella* [11]. Certain commercially available tests for the detection of *E. coli* O157 and *Salmonella* show cross-reactivity with *Aeromonas hydrophila* and/or *Citrobacter freundii* (personal communications from sprout growers). To test developed IMB-TRF protocol for cross-reactivity, the experiment described above was repeated by replacing *E. coli* O157 and *Salmonella* with *Aeromonas hydrophila* (ATCC 7965) and *Citrobacter freundii* (ATTC 8090). Even when present at concentrations as high as 10^6 CFU/g, no cross-reactivity was detected with either of these organisms (data not shown). Since the specificity and sensitivity remains high in our current study, the IMB-TRF pathogen detection process described in this report would be useful for the detection of *E. coli* O157 and *Salmonella*, individually or simultaneously, in sprouts.

3-4. Conclusion

According to International Sprout Growers Association (ISGA), annual human consumption of alfalfa sprouts in the United States has reached $250 million dollars [13]. Since 1995, raw alfalfa sprouts have emerged as a recognized source of foodborne illness in the United States. After investigation by the California Department of Health Services of sprout-related outbreaks due to *Salmonella* and *E. coli* O157, FDA issued its original Health Advisory on Sprouts in 1999. This advisory has been recently updated (10/02/2002) because of further sprout-related outbreaks of foodborne illness. The ISGA has taken positive steps to address this problem. For example, the sprout industry is pursuing the use of 2% calcium hypochlorite for soaking of the seeds prior to germination and growth. This intervention method has the potential to substantially reduce, but not necessarily eliminate, pathogenic microbial contamination of seeds that can be passed on to the consumer through ingestion of raw sprouts. Thus, it is desirable to develop effective technologies that can be applied to detect both *E. coli* O157 and *Salmonella* in alfalfa sprouts.

In this study, IMB-TRF technology has proved to have the potential to detect low levels of *E. coli* O157 and *Salmonella* in alfalfa seeds. The sensitivity of the developed immunoassay allows rapid detection of select pathogens even when the background microflora counts are extremely high. The technology may be applied to both whole growing sprouts or spent irrigation water as sampling sources and the results are obtained within 6 to 7 hours. Furthermore, the further development of a 96-well format would allow the described approach to be used as a high throughput screening procedure for pathogens in sprouted food products.

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